*THE BIOGENESIS OF MITOCHONDRIA, VI. BIOCHEMICAL BASIS OF THE RESISTANCE OF SACCHAROMYCES CEREVISIAE TOWARD ANTIBIOTICS WHICH SPECIFICALLY INHIBIT MITOCHONDRIAL PROTEIN SYNTHESIS**

By Anthony W. Linnane, Adrian J. Lamb, C. Christodoulou, and H. B. Lukins

BIOCHEMISTRY DEPARTMENT, MONASH UNIVERSITY, CLAYTON, VICTORIA, AUSTRALIA

Communicated by David E. Green, December 26, 1967

Protein synthesis by yeast mitochondria is inhibited both *in vivo*[1] and *in vitro*[2] by the antibiotics chloramphenicol, tetracycline, erythromycin, carbomycin, oleandomycin, and spiramycin. On the other hand, the drugs do not inhibit the cytoplasmic ribosomal protein synthesizing system of yeast.[1, 2] The concentration of any particular antibiotic required to inhibit mitochondrial development by growing yeast cells has been shown to be genetically determined and we have recently described the isolation of mutants resistant to one or more of the drugs.[3, 4] Broad groupings of cross-resistance toward the various antibiotics have been recognized in whole cells: thus a strain resistant to chloramphenicol is usually also resistant to tetracycline and a strain resistant to any one of the structurally related macrolide antibiotics erythromycin, oleandomycin, carbomycin, or spiramycin is usually also resistant to all the other members of the group.[4]

This communication reports an investigation of the biochemical basis of chloramphenicol and erythromycin resistance in a number of yeast mutants. We find that chloramphenicol resistance is due to a change in cell permeability which extends to tetracycline and thereby also accounts for the cross-resistance shown by whole cells towards these drugs. On the other hand, erythromycin resistance results from a change in the mitochondrial protein synthesizing system *per se*, a change which similarly affects the response of the mitochondrial system to the other macrolides.

Materials and Methods.—Strains and nomenclature: Three haploid strains of *Saccharomyces cerevisiae* (L410, 21-4B, D243-1A) were used in this investigation and mutants having increased levels of resistance to either chloramphenicol or erythromycin or both were derived from these strains as previously described.[3, 4] Resistance of a strain to an antibiotic which specifically inhibits mitochondrial development can be assessed by the ability of the organism to grow on a nonfermentable substrate in the presence of the antibiotic.[3] The sensitivity of a strain is defined by the lowest level of antibiotic which strongly inhibits cell growth on liquid glycerol medium; conversely the resistance of a strain is defined by the level of antibiotic at which growth on liquid glycerol medium is substantially unimpaired.

Most experiments were conducted with strain L410 (CAP-S, 0.1; ERY-S, 0.1) and three mutants derived therefrom, L414 (CAP-R, 1.5; ERY-S, 0.1), L411 (CAP-S, 0.1; ERY-R, 8), and L413 (CAP-R, 1.5; ERY-R, 8). CAP and ERY denote chloramphenicol and erythromycin and the suffixes R and S the whole cell resistance or sensitivity to the antibiotics; the number following the suffix is the concentration of antibiotic (mg/ml) in a glycerol growth medium to which the cells are either sensitive or resistant. A similar nomenclature has been adopted to describe the whole cell sensitivity or resistance to tetracycline (TET), spiramycin (SPI), carbomycin (CAR), or oleandomycin (OLE).

This nomenclature is used to describe the phenotypic characteristics of the strains; a genotypic description is given elsewhere.[3]

Growth conditions and mitochondrial studies: The various growth conditions for the strains are described elsewhere.[3, 5] The preparation of mitochondria and the *in vitro* assays of mitochondrial amino acid incorporation were as described by Lamb, Clark-Walker, and Linnane.[2]

Cell permeability: Cell permeability was assessed by measuring the uptake of antibiotic from a solution to which a mass of packed cells was added. To enable calculation of intracellular fluid volume and hence the intracellular antibiotic concentration, it was necessary to have a measure of the volume of extracellular fluid trapped in the initial packed-cell mass. The extracellular volume was equated with the space accessible to dextran (mol wt 500,000), a large molecule to which the cells are impermeable. Accordingly, either 20 ml of a 10% solution of dextran or 20 ml of an antibiotic solution at a concentration of 50–3000 μg/ml was added to 20 ml of packed cells in paired tubes, and the mixtures were shaken for 60 min at 30°C. The cells were removed by centrifugation, and the concentrations of dextran and antibiotic in the respective supernatant fluids determined. From the decrease in concentration of the dextran, the volume of the extracellular fluid introduced with the packed cells was calculated and by difference the intracellular volume was obtained. The partition of the antibiotic between intracellular and extracellular phases could then be readily calculated.

Chemical determinations: Chloramphenicol was determined by the method of Levine and Fischbarch,[6] erythromycin by the method of Kazenko *et al*,[7] and tetracycline by the method of Monastero *et al.*[8] Dextran concentrations were determined by measurement of the optical rotation of the various solutions.

Results.—Amino acid incorporation: Table 1 shows the effect of antibiotics on amino acid incorporation into protein by mitochondria isolated from strain L410 and the three derived antibiotic-resistant mutants. Mitochondria isolated from strains that are sensitive to erythromycin (L410, L414) were inhibited by 75 per

TABLE 1. *The effect of erythromycin and chloramphenicol on in vitro mitochondrial amino acid incorporation.*

Antibiotic Added (mM)	Strain Number and Antibiotic Susceptibility: L410 CAP-S ERY-S	L414 CAP-R ERY-S	L411 CAP-S ERY-R	L413 CAP-R ERY-R
	($\mu\mu$Moles C^{14}-Leucine Incorporated/Mg Protein/20 Min)			
None	65	42	75	58
	Per Cent Inhibition of Incorporation			
Chloramphenicol				
0.5	94	97	92	93
0.1	88	90	87	89
0.03	72	...	75	69
0.003	25	28	30	24
Erythromycin				
1.25	75	79	3	3
0.5	76	...	5	2
0.03	78	72	2	3
0.01	73	...	2	3
0.001	74	75	Nil	Nil

The incubation medium contained 38 mM Tris-HCl (pH 7.4), 100 mM KCl, 13 mM $MgCl_2$, 100 mM sorbitol, 1.5 mM ATP, 5 mM PEP, 30 μg (5 E.U.) of pyruvate kinase, 0.2 μc of C^{14}-leucine (40 μc/μmole) and approximately 1 mg of mitochondrial protein, in a final volume of 1 ml. Antibiotics were added as indicated. The results reported are for a single typical experiment. The levels of chloramphenicol and erythromycin to which the whole cells are sensitive or resistant are given in *Materials and Methods.*

TABLE 2. *Whole cell uptake of chloramphenicol and erythromycin.*

Strain Number and Antibiotic Susceptibility		Antibiotic Uptake — Chloramphenicol: Concentration ratio*	Chloramphenicol: μG/gm dry weight cells	Erythromycin: Concentration ratio*	Erythromycin: μG/gm dry weight cells
L410	CAP-S ERY-S	0.8	230	0.5	170
L414	CAP-R ERY-S	0.1	30	0.6	200
L411	CAP-S ERY-R	0.8	210	0.5	180
L413	CAP-R ERY-R	0.1	25	0.5	180

Cells were suspended in suitable solutions of either chloramphenicol or erythromycin to give a final concentration of 100 μg of antibiotic/ml of extracellular fluid after 1 hr. The uptake of antibiotic was determined as described in *Materials and Methods.*

* Concentration ratio is the ratio of the intracellular to extracellular antibiotic concentration after 1 hr; the accuracy of the determination of the ratio is of the order of ±0.05. The levels of chloramphenicol and erythromycin to which the whole cells are sensitive or resistant are given in *Materials and Methods.*

cent[9] with erythromycin at concentrations as low as 0.001 mM, but mitochondria isolated from the erythromycin-resistant strains (L411, L413) were unaffected by erythromycin even at concentrations as high as 1.25 mM. On the other hand, mitochondria isolated from strains which were either chloramphenicol-sensitive (L410, L411) or chloramphenicol-resistant (L413, L414) were equally sensitive to chloramphenicol over a range of concentrations varying from 0.003 to 0.5 mM.

To exclude the possibility that the erythromycin resistance of the isolated mitochondria was due to a change in the permeability of the mitochondrial membranes, amino acid incorporation by disrupted mitochondria was examined. Mitochondria were subjected to 10–20 seconds sonication or to three cycles of successive freezing and thawing; the organelles so treated sustained severe structural alterations as judged by the almost complete loss of ability to oxidize pyruvate plus malate. However, these mitochondria still retained 50–70 per cent of their amino acid incorporating activity and exhibited no change in the pattern of response to chloramphenicol and erythromycin. Similar results have also been obtained with erythromycin-resistant mutants derived from strain D243-1A, which is unrelated to strain L410. It is therefore concluded that the change in the whole cell sensitivity to erythromycin reflects a change in the mitochondrial protein synthesizing system *per se.*

Cell permeability: Whole cell resistance to chloramphenicol appears to be due to a change in the permeability of the plasma membrane. Table 2 shows that when packed cells are suspended for one hour in a solution of chloramphenicol such that the final external concentration of the drug is 100 μg/ml, the two chloramphenicol-sensitive strains (L410 CAP-S, 0.1; L411 CAP-S, 0.1) take up about 200–250 μg of chloramphenicol/gm dry weight of cells, while the two resistant strains (L413 CAP-R, 1.5; L414 CAP-R, 1.5) take up only about 25–30 μg of antibiotic/gm dry weight of cells. The uptake of chloramphenicol by both sensitive and resistant cells over a wide range of external concentrations is shown in Figure 1. It is apparent that with increasing concentration of chloramphenicol

the uptake by the resistant cells increases and eventually attains the level of 200–250 μg/gm dry weight of cells at an external concentration of approximately 1000–1500 μg/ml of medium. In the resistant strains the *in vivo* formation of the mitochondrial cytochromes and hence whole cell growth on glycerol is inhibited to the same extent at 1500 μg of chloramphenicol/ml of medium as are the

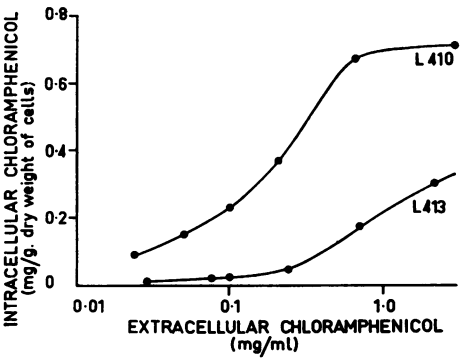

FIG. 1.—The extracellular and intracellular levels of chloramphenicol after suspension of resistant and sensitive cells for 1 hr in solutions of the antibiotic at a series of concentrations. The antibiotic susceptibilities of the whole cells are: strain L410, CAP-S, 0.1; strain L413, CAP-R, 1.5.

sensitive strains at 100 μg of chloramphenicol/ml. As amino acid incorporating activity by mitochondria isolated from both chloramphenicol-resistant and sensitive strains is equally susceptible to the antibiotic (Table 1), it is concluded that the marked difference in the cell permeability of the resistant strains adequately explains the resistance phenomenon.

Changes in whole cell permeability to chloramphenicol do not extend to erythromycin: Table 2 shows that regardless of either chloramphenicol or erythromycin resistance, all strains are equally permeable to erythromycin.

Cross-resistance to antibiotics: As detailed in Table 3, amino acid incorporation by mitochondria isolated from strain L410 is considerably inhibited by the

TABLE 3. *In vitro cross-resistance of mitochondrial amino acid incorporation to macrolide antibiotics.*

Antibiotic added (mM)	Strain Number	
	L410	L411
	(μμMoles C^{14}-Leucine Incorporated/Mg Protein/20 min)	
None	54	61
	Per Cent Inhibition of Incorporation	
Erythromycin		
0.001	74	Nil
1.25	74	3
Spiramycin		
0.03	66	1
0.50	75	2
Carbomycin		
0.03–0.1	77	6
Oleandomycin		
0.03–0.5	53	2

Mitochondria were assayed for amino acid incorporation as described in Table 1. The tabulated results are the means of five preparations. The antibiotic susceptibilities of the whole cells are: Strain L410, ERY-S, 0.1; SPI-S, 0.5; CAR-S, 0.5; OLE-0.5. Strain L411, ERY-R, 8; SPI-R, 5; CAR-R, 2; OLE-R, 2.

four macrolide antibiotics erythromycin, carbomycin, spiramycin, and oleandomycin. In contrast, mitochondria isolated from strain L411, which was initially selected for its resistance to erythromycin, were completely resistant to all four macrolide antibiotics (Table 3). A similar result was obtained with mitochondria isolated from strain L413 which was selected for erythromycin resistance, but independently of strain L411. The altered response of the mitochondrial protein synthesizing system to the macrolide group of antibiotics offers an explanation for the phenomenon of macrolide cross-resistance in whole cells.

The cross-resistance displayed by whole cells to chloramphenicol and tetracycline may be correlated with changes in cell permeability. As earlier described, the chloramphenicol sensitive strain L410 has a considerably greater permeability to chloramphenicol than has the derived resistant strain L413. Table 4 shows that strain L410 also has a greater permeability to tetracycline than strain L413. The chloramphenicol sensitive strain 21-4B and the derived resistant mutant 21-4B-1, which are completely unrelated to strains L410 and L413, also exhibit differential permeability to both chloramphenicol and tetracycline (Table 4).

It is of interest that strain L413, which is resistant to 1.5 mg of chloramphenicol/ml of growth medium, appears to exclude the drug more efficiently than does strain 21-4B-1, whose resistance is limited to 1 mg of chloramphenicol/ml of medium (Table 4). However, there was no differential exclusion of tetracycline by the two resistant strains, each of which is resistant to 1 mg of tetracycline/ml of growth medium.

Discussion.—The results presented in this paper show that in whole yeast cells there are at least two mechanisms of resistance toward antibiotics which are known to inhibit mitochondrial protein synthesis. The chloramphenicol- and tetracycline-resistant mutants described herein exhibit a specific alteration in cell permeability such that the intracellular concentration of these drugs, and hence the degree of inhibition of the mitochondrial protein synthesizing system, is reduced. The genetic determinants of cell permeability toward chloramphenicol have recently been shown to be chromosomally inherited (Saunders, Lukins, and Linnane, unpublished observations).

TABLE 4. *Whole cell permeability to chloramphenicol and tetracycline.*

Strain Number and Antibiotic Susceptibility		Antibiotic Uptake: Chloramphenicol Concentration ratio*	Chloramphenicol μG/gm dry weight cells	Tetracycline Concentration ratio*	Tetracycline μG/gm dry weight cells
L410	CAP-S TET-S	0.8	230	0.5	150
L413	CAP-R TET-R	0.1	25	0.2	60
21-4B	CAP-S TET-S	0.8	220	0.5	160
21-4B-1	CAP-R TET-R	0.2	60	0.2	70

* Experimental details are as given in the legend of Table 2. The antibiotic susceptibilities of the whole cells are: Strain L410: CAP-S, 0.1; TET-S, 0.1. Strain L413: CAP-R, 1.5; TET-R, 1. Strain 21-4B: CAP-S, 0.1; TET-S, 0.1. Strain 21-4B-1: CAP-R, 1; TET-R, 1.

In contrast, the erythromycin resistant mutants contain mitochondria with alterations in the mitochondrial protein synthesizing system, and it is of particular interest in the present context that erythromycin resistance in strains L411 and L413 is cytoplasmically inherited.[3] Erythromycin has been shown to inhibit protein synthesis in bacterial systems by interacting with the bacterial ribosome,[10, 11] and Taubman *et al.*[12] have shown that erythromycin resistance in a strain of *Bacillus subtilis* results from a change in its binding by the ribosomes. Evidence has previously been presented by this laboratory which supports the view that the yeast mitochondrial and bacterial ribosomes are similar in properties,[1, 5] and furthermore we have reported that there are specific types of high-molecular- weight mitochondrial RNA in yeast, distinct from those of the cytoplasmic ribosomal components.[13] It therefore appears likely that the erythromycin resistance described herein is associated with an alteration in the mitochondrial ribosomes. The data therefore offer suggestive evidence that the synthesis of mitochondrial ribosomes is under the control of a cytoplasmic genetic determinant which may be identical with the mitochondrial DNA. Further evidence for this view is provided by the recent experiments of Fukuhara[14] and Suyama[15] who have demonstrated specific hybridization between mitochondrial DNA and RNA but no cross-reaction between mitochondrial DNA and cytoplasmic ribosomal RNA.

Summary.—In some strains of *S. cerevisiae*, mutation to chloramphenicol and tetracycline resistance is expressed as a decrease in whole cell permeability to these drugs. In the same strains of yeast, mutation to macrolide antibiotic resistance is expressed as a change in the antibiotic sensitivity of the protein synthesizing system of mitochondria. Erythromycin resistance in the present group of mutants is extrachromosomally inherited and the interpretation is made that the synthesis of yeast mitochondrial ribosomes is under the control of a cytoplasmic genetic determinant which may prove to be mitochondrial DNA.

* This work was supported by the U.S. Public Health Service grant GM-10496-05.

[1] Clark-Walker, G. D., and A. W. Linnane, *Biochem. Biophys. Res. Commun.*, **25**, 8 (1966).

[2] Lamb, A. J., G. D. Clark-Walker, and A. W. Linnane, *Biochim. Biophys. Acta*, submitted.

[3] Linnane, A. W., G. W. Saunders, E. B. Gingold, and H. B. Lukins, these PROCEEDINGS, **59**, 903 (1968).

[4] Wilkie, D., G. Saunders, and A. W. Linnane, *Genet. Res. Camb.*, **10**, 199 (1967).

[5] Clark-Walker, G. D., and A. W. Linnane, *J. Cell Biol.*, **34**, 1 (1967).

[6] Levine, J., and H. Fischbarch, *Antibiot. Chemotherapy*, **1**, 59 (1951).

[7] Kazenko, A., O. J. Sorenson, M. C. Wolf, W. A. Dill, M. Galbraith, and A. J. Glazko, *Antibiot. Chemotherapy*, **7**, 410 (1957).

[8] Monastero, F., J. A. Means, T. C. Grenfell, and F. H. Hedger, *J. Am. Pharm. Assoc.*, **40**, 241 (1951).

[9] Inhibition by erythromycin has not been observed to exceed about 75%. This phenomenon is discussed in reference 2.

[10] Vazquez, D., *Symp. Soc. Gen. Microbiol.*, **16**, 169 (1966).

[11] Wolfe, A. D., and F. E. Hahn, *Biochim. Biophys. Acta*, **95**, 146 (1965).

[12] Taubman, S. B., N. R. Jones, F. E. Young, and J. W. Corcoran, *Biochim. Biophys. Acta*, **123**, 438 (1966).

[13] Rogers, P. J., B. N. Preston, E. B. Titchener, and A. W. Linnane, *Biochem. Biophys. Res. Commun.*, **27**, 405 (1967).

[14] Fukuhara, H., these PROCEEDINGS, **58**, 1065 (1967).

[15] Suyama, Y., *Biochemistry (Tokyo)*, **6**, 2839 (1967).